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DNA Processing Proteins Involved in the UV-Induced Stress Response of *Sulfolobales*

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ABSTRACT

The *ups* operon of *Sulfolobus* species is highly induced upon UV stress. Previous studies showed that the pili encoded by this operon are involved in cellular aggregation, which is essential for subsequent DNA exchange between cells, resulting in homologous recombination. The presence of this pilus system increases the fitness of *Sulfolobus* cells under UV light-induced stress conditions, as the transfer of DNA takes place in order to repair UV-induced DNA lesions via homologous recombination. Four conserved genes (*saci_1497* to *saci_1500*) which encode proteins with putative DNA processing functions are present downstream of the *ups* operon. In this study, we show that after UV treatment the cellular aggregation of strains with *saci_1497*, *saci_1498*, and *saci_1500* deletions is similar to that of wild-type strains; their survival rates, however, were reduced and similar to or lower than those of the pilus deletion strains, which could not aggregate anymore. DNA recombination assays indicated that *saci_1498*, encoding a ParB-like protein, plays an important role in DNA transfer. Moreover, biochemical analysis showed that the endonuclease III encoded by *saci_1497* nicks UV-damaged DNA. In addition, RecQ-like helicase Saci_1500 is able to unwind homologous recombination intermediates, such as Holliday junctions. Interestingly, a *saci_1500* deletion mutant was more sensitive to UV light but not to the replication-stalling agents hydroxyurea and methyl methanesulfonate, suggesting that Saci_1500 functions specifically in the UV damage pathway. Together these results suggest a role of Saci_1497 to Saci_1500 in the repair or transfer of DNA that takes place after UV-induced damage to the genomic DNA of *Sulfolobus acidocaldarius*.

IMPORTANCE

***Sulfolobales* species increase their fitness after UV stress by a UV-inducible pilus system that enables high rates of DNA exchange between cells. Downstream of the pilus operon, three genes that seem to play a role in the repair or transfer of the DNA between *Sulfolobus* cells were identified, and their possible functions are discussed. Next to the previously described role of UV-inducible pili in the exchange of DNA, we have thereby increased our knowledge of DNA transfer at the level of DNA processing. This paper therefore contributes to the overall understanding of the DNA exchange mechanism among *Sulfolobales* cells.**

In all domains of life, DNA repair is crucial for maintenance of genome integrity upon exposure to intra- or extracellular DNA-damaging threats (1). Unlike DNA repair in bacteria, archaeal DNA repair and its regulation are still far from well understood (1, 2). Homology searches revealed that the proteins in archaeal DNA repair pathways show similarities to both bacterial and eukaryotic proteins. In addition, DNA repair proteins with unique archaeal features exist (3).

Previously, it was speculated that archaea might have SOS-like responses similar to those in certain bacteria (4); however, microarray studies revealed that neither *Haloarchaea* (5, 6) nor *Sulfolobus* species (7, 8) show a strong induction of genes involved in excision repair upon UV-induced DNA damage. The only archaeal DNA repair genes upregulated in response to UV light are implicated to play a role in homologous recombination (HR), a pathway involved in double-strand break (DSB) repair (5–8).

For hyperthermophilic organisms such as *Sulfolobus*, DNA repair mechanisms are probably of extreme importance, as the rates of spontaneous DNA mutations are elevated at high temperatures (9). This means that DNA lesions are also probably formed more frequently. However, the mutation rates observed in the hyperthermophilic archaeon *Sulfolobus acidocaldarius* are similar to those observed in mesophilic organisms (10). Thus, efficient DNA repair systems that might involve efficient HR mechanisms seem to be present in hyperthermophilic archaea.

UV light is one of the most important causes of DNA damage in cells from all domains on Earth. It is accountable for direct DNA lesions, such as cyclobutane pyrimidine dimers (CPDs), hydrated pyrimidines, and pyrimidine-pyrimidine photoproducts, as well as for indirect lesions, such as DSBs (11). In *Sulfolobus*, large amounts of CPDs were observed directly after UV treatment (12); moreover, DSBs started to appear 2 h after UV irradiation (7). Other observed effects, probably as a consequence of DNA damage, were the general inhibition of many cellular processes and the accumulation of cells in S phase. The latter is thought to be due to the inhibition of DNA replication processes (7, 8).

Direct DNA lesions are normally repaired by base excision re-

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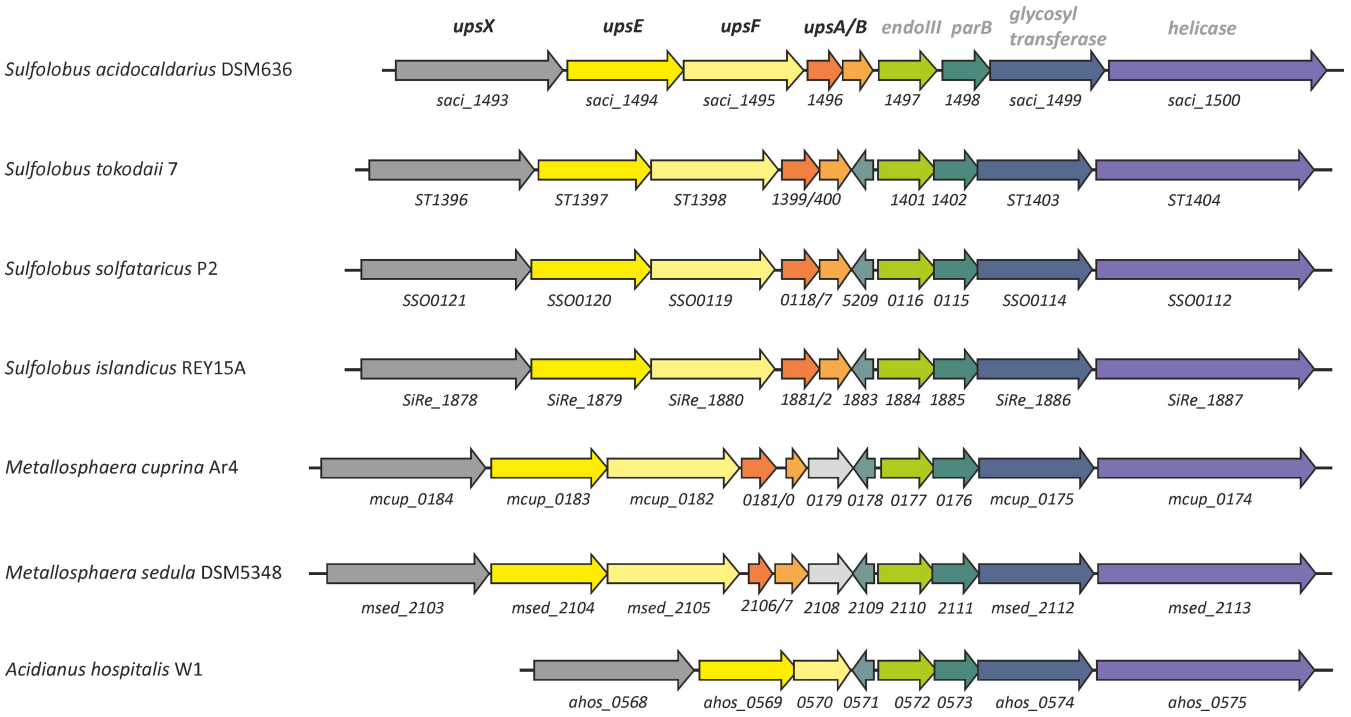

FIG 1 Schematic overview of the *ups* gene cluster and its downstream genes in different members of the *Sulfolobales*. The cluster encodes UpsX, a protein with unknown function; UpsE, an assembly ATPase; UpsF, an integral membrane protein; and UpsA and UpsB, two pilin subunits. Downstream, four genes encoding putative DNA processing proteins are present: Saci_1497, a predicted endonuclease III; Saci_1498, a ParB-like protein; Saci_1499, a predicted glycosyltransferase; and Saci_1500, a RecQ-like helicase. Homology is indicated by similar colors and was found using Absynte (31).

pair (BER) or nucleotide excision repair (NER) using the other undamaged strand as the template (11). To repair DSBs, more complex mechanisms are required. Three mechanisms are known: nonhomologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), and HR (13). Of these three, HR is considered the most accurate, given its use of an intact copy of the same DNA sequence as a template for repair. Especially in archaea, HR is presumably the major method of DSB repair, as Ku70/80 homologs involved in NHEJ are absent (14, 15). During DNA repair via HR, the ends of DSBs are processed, producing a 3′ single-stranded DNA tail that is ready for strand invasion into another undamaged copy of the homologous sequence. In eukaryotes, homologous recombination normally takes place between sister chromatids (16). Moreover, in bacteria, such as *Deinococcus radiodurans*, HR can occur with one of the multiple copies of its genome; they thereby accomplish a high level of resistance to radiation (17). As *Sulfolobus* cells are predominantly present in G_2 phase (18), most of the time there is a second copy of the chromosome to repair DSBs via HR. However, in G_1 phase or upon severe DNA damage, HR might not be possible, and unrepaired DSBs become lethal.

Sulfolobus cells exchange DNA in a UV-inducible manner (7, 19, 20). This process is mediated by cellular aggregation, in which UV-inducible pili (*ups* pili) play an essential role (21, 22). It was shown that transferred DNA can be used as a template for HR (19). Importantly, upon UV induction, *Sulfolobus* strains that have the ability to exchange DNA show significantly higher survival rates than strains that do not (19). Given the DNA-damaging properties of UV light and because the DNA-damaging agent bleomycin also induces cellular aggregation (19), we assume that the transfer of DNA between *Sulfolobus* cells is involved in DSB repair in a recombinational manner.

Downstream of the gene cluster encoding the *ups* pili, four conserved genes are present. These genes encode a predicted endonuclease III (EndoIII; Saci_1497), a ParB-like nuclease (Saci_1498), a glycosyltransferase (Saci_1499), and a DNA helicase (Saci_1500) (Fig. 1). Given the putative DNA processing functions of three of these proteins, we hypothesized that they are involved in the DNA transfer or homologous recombination taking place as soon as DNA is transferred. Here, we therefore studied Saci_1497 to Saci_1500 and their roles in the DNA repair taking place subsequent to the formation of UV-induced DNA damage. Our results show a clear link between these genes and UV-damaged DNA repair and suggest a role of the individual genes in the DNA damage response or homologous recombination. Similar to the SOS response in certain bacteria, the *ups* system in combination with homologous recombination might rescue *Sulfolobus* cells from DNA-damaging threats.

MATERIALS AND METHODS

Culture conditions. *Sulfolobus acidocaldarius* strains MW001 (23), MR31 (24), and JDS183 (25) and mutants derived from those strains (Table 1) were grown aerobically at 75°C in basic Brock medium (26) supplemented with 0.1% enzymatic digest of casein (N-Z-Amine), 0.2% dextrin, and 10 μg/ml uracil and adjusted to pH 3.5 with sulfuric acid. For plates with solid medium, the medium was supplemented with 0.6% Gelrite polymer, 3 mM $CaCl_2$, and 10 mM $MgCl_2$. Plates were incubated for 5 to 7 days at 75°C. Competent *Escherichia coli* DH5α, ER1821 (NEB), and Rosetta (DE3)pLysS (Novagen) cells, used for cloning, methylation of plasmid DNA, and protein overexpression, respectively, were grown in at 37°C LB medium (10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl) sup-

TABLE 1 Strains used during this study

Strain	*S. acidocaldarius* background strain	Genotype	Plasmid used	Reference or source
JDS183	DSM639	Δ*pyrE* (duplicate bp 44)		25
MR31	DSM639	Δ*pyrE* (deletion of bp 153–171)		24
MW001	DSM639	Δ*pyrE* (deletion of bp 91–412)		23
MW109	MW001	Δ*upsE*	pSVA1804	23
MW111	MW001	Δ*saci_1497*	pSVA1807	This study
MW122	MW001	Δ*saci_1498*	pSVA1808	This study
MW141	MR31	Δ*saci_1497*	pSVA1807	This study
MW148	JDS183	Δ*saci_1497*	pSVA1807	This study
MW149	JDS183	Δ*saci_1498*	pSVA1808	This study
MW150	MW001	Δ*saci_1500*	pSVA1835	This study
MW512	MR31	Δ*saci_1498*	pSVA1808	This study
MW514	MR31	Δ*saci_1500*	pSVA1835	This study
MW516	JDS183	Δ*saci_1500*	pSVA1835	This study
MW737	MW109	Δ*upsE* Δ*saci_1500*	pSVA1835	This study
MW718	MW001	C-terminal HA tag on *saci_1500* in genome	pSVA2634	This study

plemented with the appropriate antibiotics (100 mg/ml ampicillin, 50 mg/ml kanamycin, and/or 30 mg/ml chloramphenicol). For growth curves, a fresh culture with an optical density (OD) at 600 nm (OD_{600}) of about 0.2 was diluted to a theoretical OD of 0.002 in 50 ml fresh medium. Growth was monitored by OD_{600} measurements.

Deletion and tagging of genes in *S. acidocaldarius*. To construct deletion strains, up- and downstream flanking regions of the genes of interest (400 to 600 bp) were amplified with the primers listed in Table S1 in the supplemental material. For insertion of the tags, up- and downstream regions of the location for insertion were amplified with primers including the sequence encoding a hemagglutinin (HA) tag (these primers are also listed in Table S1 in the supplemental material). Primers were designed according to the genomic sequence of *Sulfolobus acidocaldarius* DSM 639. Overlap PCR was performed using the two outer primers to connect the up- and downstream fragments. The PCR product was subsequently cloned into pSVA406, which contains the *pyrEF* cassette from *S. solfataricus* (23), resulting in the plasmids summarized in Table 2. The plasmids were methylated by transforming them into *E. coli* ER1821 containing pM.EsaBC4I (NEB) (27). Transformation of the plasmids into different *S. acidocaldarius* background strains and the subsequent generation of mutants were performed as described previously (23). The colonies obtained were tested by PCR for successful deletion of the genes or addition of the tag. The correctness of the strains was confirmed by PCR using primers binding outside the flanking regions used for the construction of the mutant and DNA sequencing (see Table S1 in the supplemental material). The strains with deletions are listed in Table 1.

Assays for survival rates after UV treatment and aggregation experiments. UV light treatment was performed as described by Frols et al. (21); 10 ml culture (OD_{600}, 0.2 to 0.3) was treated with a UV dose of 75 J/m^2 (254 nm; Spectroline UV cross-linker) in a plastic petri dish. Afterwards, the cultures were incubated at 75°C for 3 h. Samples taken before and after treatment with UV were analyzed by phase-contrast microscopy and survival rate assays. To quantify aggregated cells 3 h after induction with UV, 5 μl of cell culture (diluted to an OD of 0.2) was spotted on a

TABLE 2 Plasmids used during this study

Plasmid	Backbone	Description	Restriction enzymes used for cloning	Primers	Reference or source
pCMalLacS	pRN1	Shuttle vector with *S. solfataricus lacS* reporter gene			74
pCMal	pCMalLacS	Empty pCMal vector	NcoI/EagI		This study
pSA4	pET15b	Expression plasmid			28
pSVA406		Backbone of deletion plasmids			23
pSVA1804	pSVA406	Used for deletion of *upsE*	ApaI/BamHI		23
pSVA1807	pSVA406	Used for deletion of *saci_1497*	ApaI/BamHI	2034–2037	This study
pSVA1808	pSVA406	Used for deletion of *saci_1498*	ApaI/BamHI	2040–2043	This study
pSVA1825	pSVA2047	Used for expression of Saci_1500 K60A in *S. acidocaldarius*		3005/3006	This study
pSVA1835	pSVA406	Used for deletion of *saci_1500*	ApaI/BamHI	2050/3048 and 3049/2053	This study
pSVA2034	pSA4	Used for overexpression of Saci_1497 in *E. coli*	NcoI/BamHI	1317/1333	This study
pSVA2038	pSA4	Used for overexpression of Saci_1500 in *E. coli*	NcoI/BamHI	1314/1315	This study
pSVA2047	pCMalLacS	Used for expression of Saci_1500 in *S. acidocaldarius*	NcoI/BamHI	1314/1315	This study
pSVA2600	pSVA2038	Used for overexpression of Saci_1497 K130A		1358/1359	This study
pSVA2601	pSVA2038	Used for overexpression of Saci_1497 D148A		1360/1361	This study
pSVA2602	pSVA2600	Used for overexpression of Saci_1497 K130A, D148A		1360/1361	This study
pSVA2612	pSVA2038	Used for overexpression of Saci_1500 K60A		3005/3006	This study
pSVA2634	pSVA406	Used to genomically insert a C-terminal HA tag on *saci_1500*	NdeI/BamHI	4606–4609	

microscope slide and covered with a thin layer of 1% agarose in Brock minimal medium. A coverslip was added when the drop had dried. Cells were visualized by phase-contrast microscopy. Free and aggregated (≥3) cells were counted for at least three fields per strain using ImageJ cell counter software. The percentages of cells in aggregates and the average amount of cells in one aggregate were subsequently calculated. For survival rate assays, serial 1:10 dilutions of the cultures were obtained and plated. To quantify the survival of cells, colonies from UV-induced and non-UV-induced cells on these plates were counted. Percentages of survival upon UV treatment and standard deviations were calculated from at least three independent experiments.

qPCR of *S. acidocaldarius* cDNA. To compare the expression of genes in and downstream of the *ups* operon with and without UV treatment, total RNA was isolated from 10 ml strain MW001 cultures (which had been treated with UV as described above) using a TriFast system (Peqlab). DNA was subsequently degraded by incubating the RNA with DNase I (RNase free; Fermentas) according to the manufacturer's protocol. Proper DNA degradation was confirmed by performing a PCR of the RNA with primer pair 2033/2087 (see Table S1 in the supplemental material). cDNA synthesis was then performed with 1 μg of RNA with a first-strand cDNA synthesis kit (Fermentas). Random primers were used, and the manufacturer's protocol was followed. Quantitative PCR (qPCR) was performed using Maxima SYBR green–carboxy-X-rhodamine qPCR master mix in a Rotor-Gene Q qPCR machine (Qiagen). qPCR primers were designed for *saci_1494* and *saci_1497* to *saci_1500*, and the melting temperatures were about 60°C. PCR with primers 2073 to 2090 (see Table S1 in the supplemental material) gave products of 80 to 150 bp in length. As a control, primers 1480 and 1481 were used for the housekeeping gene *secY*. The threshold cycle (C_T) values obtained were used to compare the non-UV-induced expression with the UV-induced expression of the tested genes. Moreover, expression by strain MW001 and the deletion strains was compared. Differences in expression are displayed as $\log_2$ fold.

DNA transfer assays. To assay DNA exchange between *S. acidocaldarius* and the derived mutants, uracil-auxotrophic strains based on MR31 (24) and JDS183 (25) were used. MR31 contains a 31-bp deletion in *pyrE*, and JDS183 contains a nonoverlapping duplication causing a frameshift in *pyrE* (25). UV treatment of the different strains was performed as described above; subsequently, all cells were brought to the same OD_{600} of 0.2. Recombination between the strains indicated below was performed by mixing different combinations of strains and plating them on plates without uracil to select for recombination events, as was described previously (19). The results of each experiment were normalized by taking the result for a mixture of two wild-type (wt) strains induced with UV light, MR31 [wt1 (UV)] and JDS183 [wt2 (UV)], which was given a value of 100%.

Expression and purification of recombinant proteins. The genes encoding Saci_1497 and Saci_1500 were amplified using *S. acidocaldarius* MW001 genomic DNA as the template and primer pairs 1317/1333 and 1314/1315, respectively (see Table S1 in the supplemental material). The PCR products were cloned into expression vector pSA4 (28), resulting in pSVA2034 and pSVA2038, respectively (Table 2). Recombinant His-tagged protein and site-directed mutants were expressed in *E. coli* strain Rosetta (DE3)pLysS (Novagen) grown in 1 liter LB medium containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) supplemented with 0.5% glucose. Cells were grown at 37°C until they reached an OD_{600} of 0.8, and expression was subsequently induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2.5 h at 37°C. Cells overexpressing Saci_1497 were harvested by centrifugation and disrupted by sonication in 500 mM NaCl, 50 mM Tris-HCl, pH 8.0. Cells overexpressing Saci_1500 were treated in the same way in the same buffer containing 200 mM NaCl. Both lysates were heat treated at 70°C for 30 min and centrifuged at 10,000 × *g* for 15 min. The heat-resistant fractions were precipitated with 80% saturated ammonium sulfate, resuspended in 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, and loaded onto a 1-ml nickel-nitrilotriacetic acid-agarose column (Sigma) which had been preequilibrated with the same buffer containing 5 mM imidazole. The columns were washed with 10 column volumes of the same buffer containing 50 mM imidazole and eluted with 3 column volumes of elution buffer containing 300 mM imidazole. Elution fractions of Saci_1497 were pooled and dialyzed against 100 mM NaCl, 50 mM Tris-HCl, pH 8.0. Saci_1500 elution fractions were loaded onto a heparin column equilibrated in 100 mM NaCl, 50 mM Tris-HCl, 2.5 mM $MgCl_2$, pH 8.0. The protein was eluted with a gradient of NaCl, using 1 M NaCl, 50 mM Tris-HCl, pH 8.0, as the maximum salt concentration. Pure Saci_1500 was dialyzed against 100 mM NaCl, 50 mM Tris-HCl, pH 8.0.

Endonuclease activity assays. pUC19 plasmid DNA was used to test specific cleavage by Saci_1497. The reaction mixture (20 μl) contained 25 mM Tris-HCl (pH 8.0), 100 mM NaCl, 500 ng (2 pmol) plasmid DNA treated with different doses of UV, and 0 to 100 nM Saci_1497. The mixture was incubated at 60°C for 40 min. The reactions were stopped by the addition of 10 μl of stop buffer containing 20 mM protease K, 50 mM EDTA, 0.5% SDS, 25% glycerol, and 0.025% bromophenol blue. The mixtures were then incubated at room temperature for 10 min and loaded onto a 0.8% agarose gel. The bands were quantified with ImageJ software.

DNA binding and helicase assays. To create different DNA substrates for binding and helicase assays, oligonucleotides A to H (see Table S1 in the supplemental material) (29) were purchased from Sigma. With these oligonucleotides and by use of the method described previously (30), the following DNA substrates were prepared and purified: double-stranded DNA (dsDNA) with a 5′ overhang, dsDNA with a 3′ overhang, blunt dsDNA, Holliday junction (HJ) DNA, and Y-shaped DNA.

Standard DNA binding assays were performed with a 20-μl mixture containing 25 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM magnesium chloride, 1 mM dithiothreitol (DTT), 1× bovine serum albumin (BSA; NEB), 5 nM DNA substrate, 10% glycerol, and the amount of enzyme indicated below. The mixtures were incubated at 60°C for 25 min and loaded on a 6% polyacrylamide gel in TBE (45 mM Tris-borate, 1 mM EDTA) buffer. Samples were electrophoresed at 100 V for 60 min. The gels were scanned using a Typhoon Storm 840 scanner (GE Healthcare), and the bands representing the unbound DNA were quantified using ImageQuant software.

Helicase activity assays were carried out in a 20-μl mixture containing 25 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM magnesium chloride, 1 mM DTT, 1× BSA (NEB), 4 mM ATP, 5 nM DNA substrate, and the concentration of enzyme indicated below. The mixtures were incubated at 60°C for 30 min, and the reactions were stopped by the addition of 10 μl stop buffer containing 20 mM protease K, 50 mM EDTA, 0.5% SDS, 25% glycerol, and 0.025% bromophenol blue. The mixtures were then incubated at room temperature for 10 min, loaded onto a 10% polyacrylamide gel in TBE buffer, and electrophoresed at 100 V for 90 min. The gels were scanned using the Typhoon Storm 840 scanner (GE Healthcare).

Spot assays with DNA-damaging agents. Wild-type and deletion strains were grown to an OD_{600} of 0.2. Dilution series were made, and 10 μl of diluted cultures with, theoretically, 1.0×10^6, 1.0×10^5, 1.0×10^4, 1.0×10^3, and 1.0×10^2 cells was spotted onto the plates. The plates contained either no additional compound, 1 mM hydroxyurea (HU), or 3 mM methyl methanesulfonate (MMS). Growth was evaluated after 7 days of growth at 75°C.

RESULTS

Transcriptional response to UV of *saci_1497* to *saci_1500*. Bioinformatic analysis using the Absynte web tool (31) revealed that all species with a UV-inducible pilus (*ups*) operon, all of which are members of the *Sulfolobales* (Fig. 1), contain four conserved genes (*saci_1497* to *saci_1500* in *S. acidocaldarius*) downstream of this cluster. These encode a putative endonuclease III, a ParB-like protein, a glycosyltransferase, and a RecQ-like helicase, respectively. We therefore speculated about a potential role of the proteins encoded by these genes that is linked to the function of the *ups* pili. For that reason, the transcription patterns of genes in

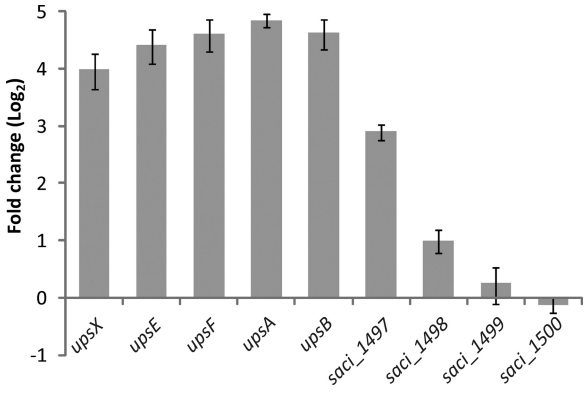

FIG 2 qPCR analysis of expression levels upon UV treatment. The changes in the levels of *saci_1493* to *saci_1500* expression in strain MW001 at 3 h after UV treatment (75 J/m^2) are shown. Differences are displayed as $\log_2$ fold changes. The primers used are summarized in Table S1 in the supplemental material.

and downstream of the *ups* operon upon UV stress were analyzed by qPCR experiments carried out with cDNA isolated from UV-induced *S. acidocaldarius* MW001 cells. The complete *ups* operon was shown to be highly upregulated upon treatment with UV (Fig. 2), confirming the findings of previous microarray studies (7, 8). Of the genes downstream of the *ups* operon, *saci_1497* showed significant upregulation (Fig. 2), suggesting a role in the UV response of *S. acidocaldarius*. Given the fact that we previously did not observe any readthrough between *upsB* and *saci_1497* in operon mapping experiments (22), it seems that Saci_1497 has its own UV-inducible promoter. The other three genes showed little to no induction after UV treatment. Using a strain with genomically tagged Saci_1500, we could additionally confirm at the protein level that there was no induction of Saci_1500 upon UV treatment (see Fig. S1 in the supplemental material).

Deletion mutant analysis. To study the role of the *saci_1497* to *saci_1500* genes in more detail, mutants with the markerless deletion of *saci_1497*, *saci_1498*, and *saci_1500* were created as described previously (23). No mutant with a deletion of *saci_1499* could be obtained using our standard deletion method, suggesting an essential role of this gene under normal growth conditions. Mutants with deletions of the other three genes were successfully created (Table 1). Both the Δ*saci_1497* and Δ*saci_1500* strains showed normal growth compared to that of strain MW001; the Δ*saci_1498* deletion strain, on the other hand, had a slightly delayed growth and smaller colonies compared to the time to growth and colony size of the MW001 background strain (data not shown).

All three deletion strains showed UV-induced aggregation that was similar to that of wild-type *S. acidocaldarius* (MW001) (Fig. 3A), which indicated that the *ups* pili were normally formed. However, upon UV irradiation, the survival rates of all three strains were found to be significantly lower than that of MW001 (Fig. 3B). For the Δ*saci_1497* and Δ*saci_1498* mutants, this reduction in survival rate was found to be comparable to that of the previously described Δ*upsE* strain, which could not assemble *ups* pili (19). One could speculate that even though the Δ*saci_1497* and Δ*saci_1498* deletion strains were still able to form pili and aggregates, they could no longer transfer DNA or perform efficient DNA repair in order to repair DNA lesions, resulting in lower survival frequencies. The Δ*saci_1500* strain showed even lower survival rates after UV treatment, which might be due to the fact that Saci_1500 additionally functions in other processes. This idea is emphasized by the fact that a double mutant with both *upsE* and *saci_1500* mutations had survival rates that were slightly lower than those of a single *saci_1500* mutant (Fig. 3B; see also Fig. S2 in the supplemental material).

To study the possible role of Saci_1497, Saci_1498, and Saci_1500 in the exchange of DNA between *Sulfolobus* cells, DNA exchange was assayed as described previously (19). Two different background strains containing nonoverlapping *pyrE* mutations were used (Table 1). Upon DNA transfer between these two strains, homologous recombination could give rise to an intact *pyrE* gene, leading to the formation of recombinants on plates without uracil (Fig. 3C; see also Table S2 in the supplemental material). As was shown before (19), a mixture of two *ups* wild-type strains resulted in the formation of recombinants (Fig. 3C, wt1-wt2, green bar), indicating DNA exchange between the two strains. Moreover, the induction of one or both strains with UV light resulted in significantly higher recombination frequencies (Fig. 3C, wt1-wt2, red bars).

Mixing of the Δ*saci_1497* or Δ*saci_1500* strain with a wild-type strain or the respective mutant in the other background resulted in recombination frequencies similar to those of the wild-type mixtures (Fig. 3C, 2nd, 4th, 5th, and 7th mixtures), implying that the predicted endonuclease (Saci_1497) and helicase (Saci_1500) do not have a direct role in DNA transfer. Mixtures with the Δ*saci_1498* strain, on the other hand, had significantly reduced recombination frequencies (Fig. 3C, 3rd mixture); this effect was found to be even more drastic when two Δ*saci_1498* mutants were mixed with each other (Fig. 3C, 6th mixture). Given the fact that the exchange of DNA between two Δ*saci_1498* mutants was not completely abolished, we cannot say that Saci_1498 is essential for DNA transfer; the protein does, however, seem to play an important role. It might therefore be involved in processes such as preparation of the DNA for transfer or binding and directing incoming DNA. The reduced recombination frequencies might also account for the reduced survival rates of the Δ*saci_1498* strain. If and how Saci_1497 and Saci_1500 also play a direct role in the UV-damage response remain to be shown.

Saci_1497 cleaves UV-treated DNA. To get more insights into the possible function of Saci_1497, prediction of its structure with the SMART modular architecture research tool was performed (32). The results revealed the presence of two helix-hairpin-helix domains (HhH) and a C-terminal iron-sulfur cluster loop (FCL). These domains are typical for endonuclease III proteins; for that reason, Saci_1497 is also annotated as an endonuclease III. Endonuclease III proteins are apurinic/apyrimidinic (AP) endonucleases with an associated *N*-glycosylase activity specific for a number of hydrated, oxidized, or ring-fragmented pyrimidine residues that function in base excision repair (BER) (33–35). EndoIII from *E. coli* was first identified to be a protein with cleavage activity toward X-irradiated DNA (36) and was subsequently purified by using heavily UV-irradiated DNA as the substrate (37).

To study the putative DNA processing functions of *S. acidocaldarius* EndoIII (Saci_1497) *in vitro*, His-tagged Saci_1497 was overexpressed in *E. coli* and purified (see Fig. S2 in the supplemental material). Given the fact that bacterial and eukaryal EndoIII proteins are able to process UV-damaged DNA and introduce DNA strand nicks (38–41), we also tested the cleavage activity of Saci_1497 using pUC19 plasmid DNA as the substrate. From pre-

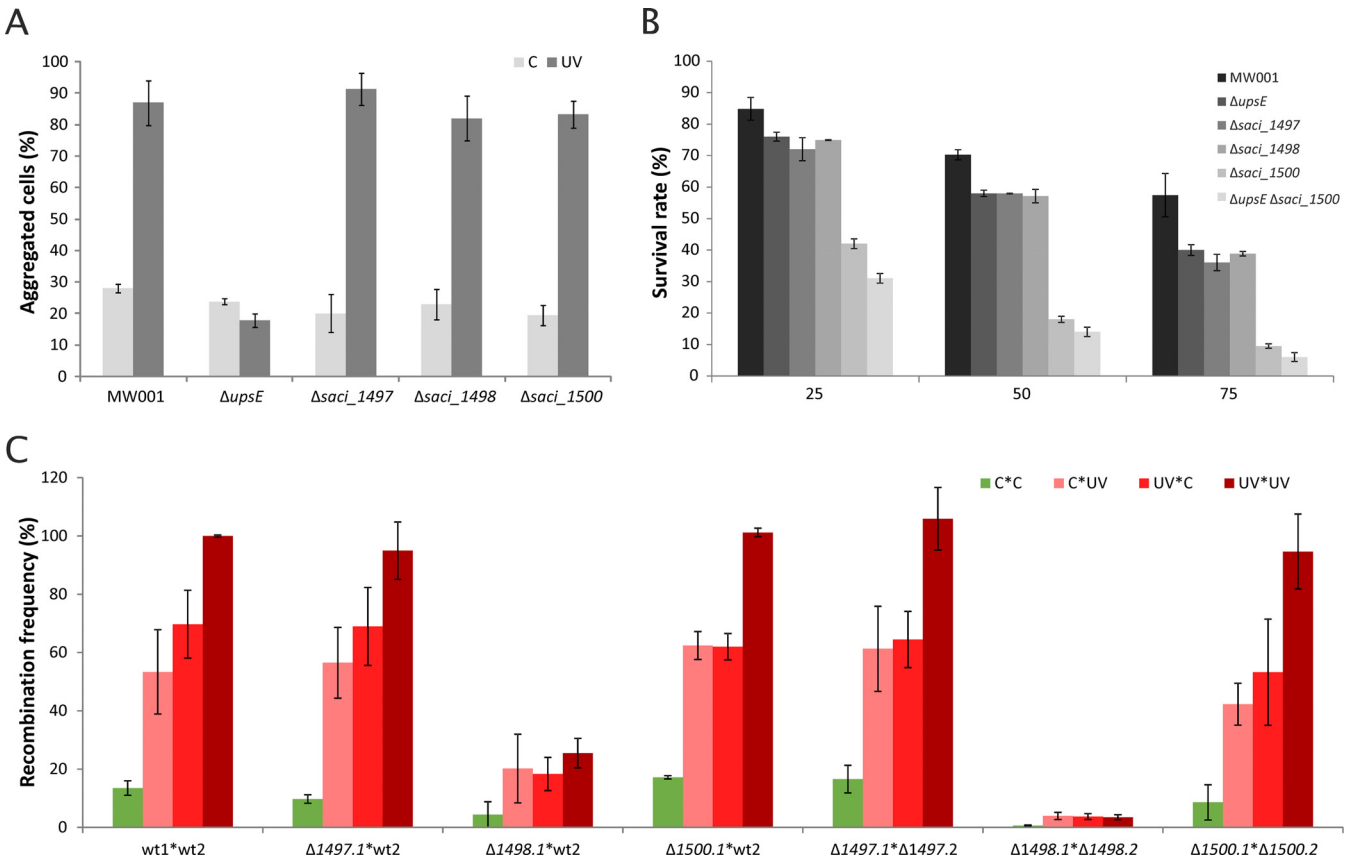

FIG 3 Analysis of deletion mutants of genes downstream of the *ups* operon. These experiments used the Δ*saci_1497*, Δ*saci_1498*, Δ*saci_1500*, Δ*upsE*, and Δ*upsE* Δ*saci_1500* strains. (A) Percentage of cells in aggregates without UV induction (as a control [C]) or with UV induction (75 J/m^2) (UV). Strains for this experiment were made in the strain MW001 background (Table 1). (B) Survival frequencies of the deletion strains (in the strain MW001 background) after treatment with UV (25, 50, or 75 J/m^2). The results for untreated strains were taken as 100%, and the survival frequencies upon treatment with UV were calculated accordingly. (C) Recombination frequencies of experiments with mating between *S. acidocaldarius* wild-type and mutant strains. The background strains used in these experiments were MR31 (wt1) (Table 1) and JDS183 (wt2) (Table 1). Two strains were mixed together in different combinations and plated on selective medium. Each of the strains was not treated with UV light (as a control) or was treated with 75 J/m^2 UV light (UV). All strains contained mutations at different positions in the *pyrE* gene (involved in *de novo* uracil biosynthesis), such that recombination between two strains could restore the wild-type phenotype. Each bar represents the average from 3 independent mating experiments, and the result of every experiment was normalized to that for the wt1 (UV)-wt2 (UV) combination, which was given a value of 100%.

vious studies, we know that UV-C light causes 2.45 cyclobutane pyrimidine dimers and 0.56 photoproducts per J/m^2 UV-C light per 10^8 Da of DNA (42). Using this information, we calculated that 1 kJ/m^2 of UV-C light is expected to cause about 41 cyclobutane dimers and 9 photoproducts per molecule of plasmid DNA. For cyclobutane pyrimidine dimers, the ratio of T-T/C-T/T-C/C-C dimers after induction with such a dose is 68:13:16:3 (42), meaning that in this experiment, about 28 T-T, 5 C-T, and 6 T-C dimers and 1 C-C dimer were formed per plasmid molecule. As shown in Fig. 4A, Saci_1497 showed no activity toward regular plasmid DNA but did introduce nicks into UV-treated plasmid DNA in a dose-dependent manner. Thereby, supercoiled plasmid DNA was converted into open circular DNA (Fig. 4B). On the basis of quantification of the data in Fig. 4B, about 50% of supercoiled substrate was converted to open circular DNA when treated with 1 kJ/m^2. These data are similar to those observed for *E. coli* EndoIII, which also targets minor forms of UV-induced damage (38).

On the basis of structure predictions for Saci_1497 (I-TASSER server), lysine 130 and aspartate 148 form putative active sites (43). Site-directed mutants with mutation of these two residues (see Fig. S2 in the supplemental material) had only 2% and 18% of the activity of the wild-type protein, respectively, confirming the prediction of the active site (Fig. 4C and D). From this we can conclude that, similarly to EndoIII from bacteria and eukarya, archaeal EndoIII from *S. acidocaldarius* can also process UV-damaged DNA and is therefore probably directly involved in the repair of damaged DNA.

***saci_1498* encodes a ParB-like nuclease.** Given the fact that a *saci_1498* deletion mutant showed strongly reduced DNA exchange (Fig. 3C, 3rd and 6th mixtures) and because Saci_1498 is predicted to contain a ParB-like nuclease domain (32), we assumed that Saci_1498 plays a role in DNA processing activities related to the transfer of DNA. To perform DNA binding and nuclease assays, we successfully overexpressed and purified Saci_1498 from *E. coli*. Unfortunately, the protein partially aggregated during our assays and we could not confirm any binding of Saci_1498 to DNA or any activity of Saci_1498 toward DNA (data not shown).

Saci_1500 shows a typical RecQ-like helicase activity. Along

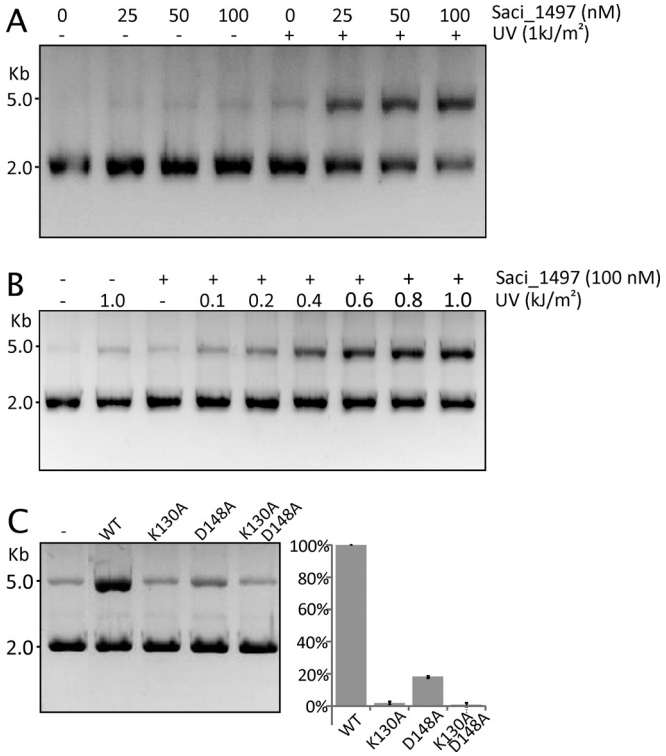

FIG 4 Activity of Saci_1497 toward UV-treated plasmid DNA. Supercoiled pUC19 plasmid DNA (500 ng) was treated with the indicated doses of UV. The DNA was subsequently incubated with the indicated concentrations of Saci_1497. Nicking activity was visualized on an agarose gel. (A) Increasing amounts of Saci_1497 were used for the assay with or without prior UV treatment of the DNA. (B) Increasing UV doses were used to treat the DNA before incubation with Saci_1497. (C) Three Saci_1497 proteins in which the active sites of the enzyme were mutated, K130, D148A, and K130A/D148A, were used to perform the same experiment described in the legends to panels A and B with a UV dose of 1 kJ/m^2 and 100 nM enzyme. Quantification of the assay whose results are shown on the left is shown on the right and was done by taking the wild-type activity as 100% and calculating the mutant activities accordingly.

with the search for biochemical insights into Saci_1497, we also tried to get more biochemical insights into Saci_1500. Given the fact that Saci_1500 is a direct homolog of Hel112, a *S. solfataricus* RecQ-like helicase that was studied before (44, 45), we assumed that Saci_1500 would function similarly. Bioinformatic analyses, including BLAST analysis (46), SMART predictions (32), and structural predictions, indeed revealed that Saci_1500 is predicted to be a typical RecQ-like helicase. RecQ helicases are capable of unwinding a variety of substrates, most of which are intermediates of the homologous recombination (HR) pathway. *In vitro*, RecQ helicases do not unwind blunt-end dsDNA. Instead, they require several nonduplex nucleotides for loading. The simplest of such structures is a single-stranded DNA (ssDNA) overhang (47, 48).

Using purified recombinant Saci_1500 (see Fig. S2 in the supplemental material), we performed binding and unwinding assays on different DNA substrates. Binding assays revealed that Saci_1500 binds with a high affinity to several DNA substrates, including HJ DNA, Y-shaped DNA, DNA with a 3′ overhang, and ssDNA. It also bound to blunt-end dsDNA but with lower affinities (Fig. 5A). DNA unwinding assays revealed that Saci_1500 does not unwind dsDNA substrates with a 5′ ssDNA overhang (Fig. 5B) or blunt-end dsDNA (Fig. 5C). dsDNA with a 3′ ssDNA overhang, Y-shaped DNA, and HJ DNA, on the other hand, were unwound efficiently (Fig. 5D to F). As a negative control, 400 nM Walker A mutant Saci_1500 K60A was used, and as expected, it showed no activity toward any of the substrates used (Fig. 5B to F, lanes C). The fact that HJ DNA was bound and also unwound with affinities similar to those of, for instance, dsDNA with a 3′ ssDNA overhang suggests a mode of substrate recognition by Saci_1500 more complicated than the simple recognition of a dsDNA-ssDNA junction.

For the direct homolog Hel112 from *S. solfataricus*, it was previously shown *in vitro* that Cy-labeled HJ substrates were mostly unwound to Y-shaped intermediates but, unlike our findings, not to ssDNA (44). When we used Cy3-labeled substrates instead of radioactively labeled DNA, we obtained similar results; in addition, we could not completely boil these substrates to ssDNA (data not shown). We therefore assume that the Cy labels cause cross-linking between DNA strands of the HJ substrate, making it impossible to show the full unwinding of the HJ substrates in the mentioned study (44), as was shown by us in Fig. 5F.

Sensitivity of Δ*saci_1500* to different DNA-damaging reagents. To confirm our *in vitro* data and emphasize the importance of the helicase activity of Saci_1500 in *vivo*, we complemented the Δ*saci_1500* deletion strain with maltose-inducible expression plasmids expressing Saci_1500 or Saci_1500 with a K60A mutation (Saci_1500 K60A; Walker A mutant) (Table 2). As positive and negative controls, we also transformed the empty vector (pCMal) into the wild-type strain MW001 and the Δ*saci_1500* deletion strain, respectively. We assayed the survival rates of all four strains upon treatment with different UV doses. The UV-sensitive phenotype of Δ*saci_1500* was almost fully complemented by pCMal-*saci_1500*, whereas the Δ*saci_1500* deletion strains transformed with either the empty plasmid pCMal or pCMal-*saci_1500* K60A were still highly sensitive to UV light (Fig. 6). Interestingly, expression of Saci_1500 K60A in a Δ*saci_1500* background resulted in survival rates even lower than those for the strain with the Δ*saci_1500* deletion alone (Fig. 6) upon UV treatment. This could be due to the fact that the substrate binding of Saci_1500 K60A without subsequent processing might hamper the activation of other pathways.

Next to UV irradiation, we also treated the Δ*saci_1500* deletion strain with two agents that stall replication in *Eukarya*: methyl methanesulfonate (MMS) and hydroxyurea (HU). Unpublished data suggest that HU also affects replication elongation in *Sulfolobus* (W. Y. Han and Q. She, personal communication), whereas MMS was reported to specifically cause reverse gyrase degradation and subsequent genomic DNA degradation (75). Strikingly, Δ*saci_1500* did not show increased sensitivity toward these agents compared to that of wild-type cells (Fig. 7), which is different from the findings for RecQ deletion mutants in the *Eukarya* (49–52). Together these results suggest that the ATPase and unwinding activity of Saci_1500 is specifically involved in UV-related DNA damage repair.

DISCUSSION

In all domains of life, HR plays an essential role in the repair of double-strand DNA breaks (DSBs) (53). As HR relies on the presence of another intact copy of the damaged DNA, it has been suggested that prokaryotes take up similar DNA from the environment to increase the chances of having a nondamaged homologous template for DNA repair (54). It has even been proposed that

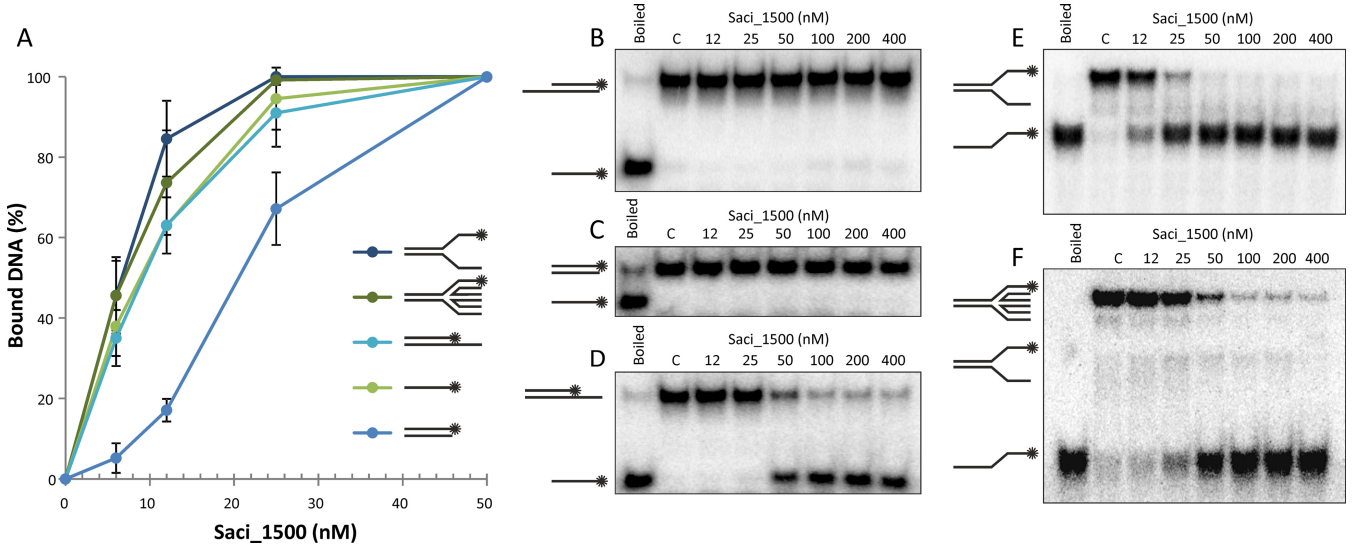

FIG 5 Activity of Saci_1500 toward different DNA substrates. (A) Binding of different concentrations of Saci_1500 to Y-shaped DNA, HJ DNA, DNA with a 3′ overhang, ssDNA, and dsDNA (from top to bottom, respectively). The binding was analyzed on a 6% polyacrylamide gel, and the gel was scanned and quantified using ImageQuant software. The percentage of bound DNA was quantified as total DNA minus the remaining free DNA substrate, and the total DNA level in the control was set to 100%. (B to F) Analysis of DNA unwinding activity of Saci_1500 toward the following substrates: DNA with a 5′ overhang (B), dsDNA (C), DNA with a 3′ overhang (D), Y-shaped DNA (E), and HJ DNA (F). Saci_1500 K60A (400 nM) was used as a control (lanes C). Boiled DNA was loaded to show the size of nonduplexed DNA (lanes Boiled). Stars represent the radioactively labeled strand in the different substrates. Gels were scanned using a Typhoon Storm 840 scanner (GE Healthcare) visualizing the radioactively labeled DNA.

DNA repair is the primary function of DNA transfer (55) and that DNA transfer might have evolved as a DNA damage response in certain bacteria lacking the SOS response (56). In line with this idea, many SOS-less bacteria induce their competence system upon DNA damage. Moreover, DNA uptake is often strongly biased toward DNA from the same species (49).

Archaeal *Sulfolobus* species also ensure the exchange of species-specific DNA by inducing their unique *ups* system (50). This DNA exchange mechanism is mediated by UV-inducible type IV pili (*ups* pili) that are able to initiate the formation of species-specific aggregates (21). Chromosomal DNA is exchanged within these aggregates, and with marker recombination frequencies of up to 10^{-2}, the system is highly efficient (19). For three important reasons, the *ups* system is proposed to be linked to DNA repair (51). First of all, aggregation takes place only between cells from the same species. DNA exchange is therefore species specific, giving rise to homologous DNA templates prone to being correct for HR (21). Second, the system is also induced upon treatment with other DNA-damaging agents, such as bleomycin (21). Lastly, the absence of the system results in reduced survival rates upon UV treatment (19). Consequently, it is expected that *Sulfolobales* exhibit a unique HR-based community DNA repair mechanism triggered by DNA DSBs and mediated by *ups* pili. This mechanism might be of great advantage for the survival of thermophilic *Sulfolobales*. Similar mechanisms have so far not been observed in other microorganisms.

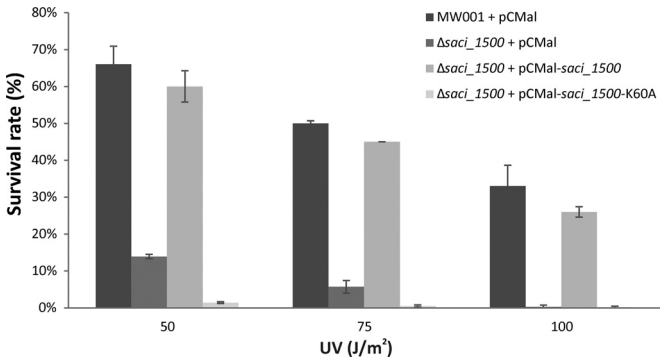

FIG 6 Survival rate assays upon UV induction of the Δ*saci_1500* and complemented strains. The following strains were treated with the indicated doses of UV: MW001/pCMal (positive control), MW001 Δ*saci_1500*/pCMal (negative control), MW001 Δ*saci_1500*/pCMal-*saci_1500* (wild-type complementation), and MW001 Δ*saci_1500*/pCMal-*saci_1500* -K60A (complementation with the Walker A mutant). The percent survival upon UV treatment was calculated from at least three independent experiments.

In archaea, operon-encoded Mre11, Rad50, HerA, and NurA are responsible for DSB end resection during HR. However, little is known about archaeal proteins involved in HR processes other than end resection (52). In this study, we investigated the role of four genes (*saci_1497* to *saci_1500*) downstream of the *ups* gene cluster encoding an endonuclease III, a ParB-like protein, a predicted glycosyltransferase, and a helicase, respectively. As *saci_1499*, encoding a putative glycosyltransferase, could not be knocked out, it was not studied in more detail. Analysis of mutants with deletion of the other three genes revealed that survival rates upon UV treatment were lower than those of wild-type cells. As mentioned before, we hypothesized that the genes are involved in DNA transfer or HR-based DNA repair, which in both cases would fit with the observed lower survival frequencies upon UV treatment. Conjugation assays, moreover, revealed that for a *saci_1498* deletion strain, DNA transfer was severely affected but not completely abolished, suggesting that Saci_1498 functions at the level of DNA transfer.

Saci_1498 is the only genomically encoded true ParB homolog in *S. acidocaldarius*. However, since the expression patterns of

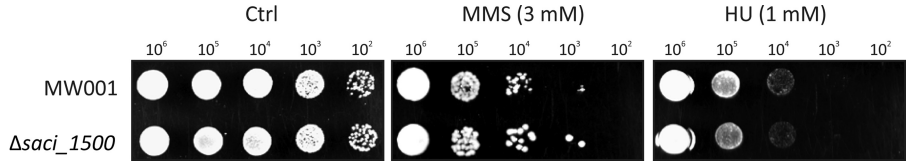

FIG 7 Sensitivity of MW001 and MW001 Δ*saci_1500* to different DNA-damaging agents. Dilution series of exponentially growing cultures were spotted onto plates with 3 mM MMS or 1 mM HU. The approximate numbers of cells per spot are indicated. As a control, plates without DNA-damaging agents were used. Similar results were obtained in at least three independent experiments.

saci_1498 are not cell cycle regulated (57) and because the growth behavior of a deletion strain is affected in only a minor way, Saci_1498 does not seem to function in DNA partitioning. For DNA-secreting gonococci, it was suggested that homologs of ParA and ParB might bind to DNA and bring it to the type IV secretion machinery (T4SS), where it is further processed by a relaxase and a helicase before it is secreted as ssDNA (58, 59). Interestingly, deletion mutants of ParA or ParB in *Neisseria gonorrhoeae* indeed showed strongly reduced DNA secretion (60, 61). We could therefore envision that Saci_1498 similarly brings the DNA to the putative transport machinery. Additionally or alternatively, it might function in the processing of DNA before transport, given its predicted nuclease domain. To characterize Saci_1498, the protein was purified from *E. coli* and used in *in vitro* experiments. However, so far no DNA binding or cleavage activity has been observed. This could be due to the absence of important cofactors or sequence-specific DNA substrates.

Moreover, we tried to biochemically characterize Saci_1497 and showed that it is able to introduce single-strand breaks in UV-damaged plasmid DNA, converting the plasmid from its supercoiled form to open circular DNA. These results clearly show that Saci_1497 on its own has activity toward damaged DNA and therefore functions in DNA repair pathways. Endonuclease III proteins are apurinic/apyrimidinic (AP) endonucleases with an associated *N*-glycosylase activity specific for a number of oxidized, hydrated, or ring-fragmented pyrimidine residues that normally function in BER (33–35). Indirectly, however, they might also be involved in HR; both in *E. coli* and in human cells it was shown that BER can lead to the formation of DSBs if two opposing damaged bases are being incised by EndoIII at the same time. Interestingly, deletion mutants of EndoIII show a reduced formation of DSBs but are more sensitive to radiation (62–66). Eccles et al. (67) discussed that repair of opposing DNA lesions by the BER pathway is slow and delayed and might induce DNA mutation or replication breakdown; however, if the opposing DNA lesions are incised only by EndoIII and not processed by the other BER proteins, a DSB which is repaired much faster occurs (67). The formation of DSBs caused by EndoIII might therefore actually be beneficial for the cells. A study of integrative phage CTXΦ infecting *Vibrio cholerae* revealed that EndoIII increases the efficiency of integration via HR by stabilizing Holliday junctions independently of its normal BER function (68). Saci_1497 might therefore also function similarly to EndoIII from *Vibrio cholerae*. We initially hypothesized that Saci_1497 might function in DNA transport, as we have now found for Saci_1498; however, the results from our conjugation assays prove otherwise.

Lastly, we characterized RecQ homolog Saci_1500. RecQ helicases are referred to as conserved guardians of genomic integrity (69) and are reported to be involved in multiple pathways for the maintenance of genomic stability, including HR (69–73). As was shown for its previously studied ortholog in *S. solfataricus* (Sso0112 or Hel112) (44, 45), we demonstrated that Saci_1500 shows typical RecQ-like DNA binding and unwinding activities. It catalyzes the processing of HR intermediates, such as Holliday junctions and Y-shaped DNA, and unwinds dsDNA with an overhang in a 3′-to-5′-dependent fashion. These results suggest a role of Saci_1500 in HR. Interestingly, however, different from the findings for eukaryal mutants of RecQ homologs BLM and Sgs1a, the *saci_1500* deletion mutant showed reduced survival rates when treated with UV light but not when treated with the replication-stalling agent HU. These results suggest that Saci_1500 processes only HR intermediates created from UV-damaged DNA and does not have a role in the processing of similar structures in stalled replication forks.

In conclusion, we have provided insights into the possible role of three genes downstream of the *ups* operon which encode an endonuclease (Saci_1497), a ParB-like protein (Saci_1498), and a RecQ-like helicase (Saci_1500). Our results suggest that these proteins are involved in processes that are (indirectly) related to the DNA transfer that is mediated by the *ups* system. Saci_1497 and Saci_1500 are proposed to function in an HR-based DNA repair mechanism that uses transferred DNA as a template. Saci_1498, on the other hand, is proposed to function directly in the DNA transfer machinery, possibly by guiding the DNA to the DNA transporter. Combining previous and current findings, we propose a DNA damage response in which the *ups* system in combination with homologous recombination rescues *Sulfolobales* from DNA-damaging threats.

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